IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Inventors:	Huber et al.
Serial No.:	09/770,410
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Examiner:	Therkorn, Ernest G.
Customer No.:	26259
Group Art Unit:	1723
Confirmation No.:	5186
Title:	Method and Apparatus for Separating Polynucleotides Using Monolithic Capillary Columns

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By _____
Typed Name: Jane Massey Licata, Reg. No. 32,257

Commissioner for Patents
Mail Stop AF
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

DECLARATION UNDER RULE § 1.131

I, Andreas Premstaller, hereby declare that:

1. I am a co-inventor, together with Christian Huber and Herbert Oberacher, in U.S. Patent Application Serial No. 09/770,410 filed June 7, 2000 and am most familiar with the subject matter of this application and the research effort which lead to the discovery of the instant invention. All the work described in the following paragraph occurred at the Institute of

AC

Analytical Chemistry and Radiochemistry in Innsbruck, Austria, a recognized WTO member country since January 1, 1995.

2. I have reviewed Gusev et al. (September 1999) *J. Chromatography* 855:273-290) and find that this reference describes a porous monolithic packing prepared with polystyrene-divinylbenzene support which is covalently attached to a fused silica capillary inner wall treated with a coupling agent trimethoxysilyl propyl methacrylate to provide anchoring sites for grafting of the polymer to the silica surface. The median pore radius for a monolithic sample prepared with ethanol is, as estimated by Gusev, about 5 micrometers.

3. Our invention referenced above, teaches a device for separating a mixture of polynucleotides by ion pair-reversed phase-high performance liquid chromatography. The device comprises a polymeric monolith having non-polar chromatographic surfaces. The monolith comprises an underivatized poly-(styrene/divinylbenzene) matrix and is contained within a tube having an inner diameter in the range of 1 to 1000 micrometers.

4. Laboratory protocol notebooks regarding experiments related to this invention were kept by me as a Ph.D. student under the direction of Christian Huber.

5. I worked in Dr. Huber's laboratory during 1998 and 1999.

6. According to laboratory protocol notebooks submitted herewith, the first synthesis of PS/DVB monolith using decanol and tetrahydrofuran as porogens was performed on August 6, 1998. We then succeeded in a first separation of proteins (lysosome from beta-lactoglobulin B) in a PS/DVB monolithic column on August 25, 1998. See, *e.g.*, the chromatograph at the bottom right-hand corner of the fourth laboratory notebook page. The first successful separation of oligonucleotides on a PS/DVB

monolith synthesized with decanol/THF as porogens was February 10, 1999.

7. We were able to fully practice our invention described in the above-referenced patent application prior to the date of the publication of the Gusev paper. A copy of the relevant laboratory notebook pages hereby accompanies my declaration.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

21.02.2006
Date

Andreas Premstaller, Ph.D.

5.8.98

Monolithsynthese mit THF M11

C10OH siehe 20.8.98 ?

Nr.	Datum	Kapillare ID/OD [µm]	Polymerisationsmischung Styrol [ml]	DVB [ml]	AIBN [g]	C12OH [ml]	THF [ml]	Temperatur [°C]
M11_1	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	3.00	0.00	70, TS
M11_2	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.90	0.10	70, TS
M11_3	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.80	0.20	70, TS
M11_4	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.70	0.30	70, TS
M11_5	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.60	0.40	70, TS

THF sollte besser Lm als Hexan sein und ähnlich als Toluol

THF destilliert, da mit Radikalfänger (Phenol) stabilisiert.

Ausgangsmaterial: VS 3.8.98 trocken
THF dest.

Start: 6.8.98 10⁰⁰h   T = 70°C

Ende: 7.8.98 12⁰⁰h   T = 70°C

7.8.98

M11_1   16 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
5	1	0.06
10	1	0.06
25	4	0.25
50	7	0.44
100	14	0.88
150	21	1.31
200	28	1.75
k [bar cm⁻¹ µl⁻¹ min]		0.008712

M11_2   15 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
10	1	0.07
25	4	0.27
50	8	0.53
100	14	0.93
150	20	1.33
200	25	1.67
k [bar cm⁻¹ µl⁻¹ min]		0.008303

M11_3   15 cm
Start constant pressure 55µl

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
10	1	0.07
25	3	0.20
50	6	0.40
100	11	0.73
150	14	0.93
200	19	1.27
k [bar cm⁻¹ µl⁻¹ min]		0.006114

M11_4   16 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
5	9	0.56
10	14	0.88
25	32	2.00
50	68	4.25
100	126	7.88
150	180	11.25
k [bar cm⁻¹ µl⁻¹ min]		0.074602

M11_5   16 cm   200bar Gegendruck

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
1	21	1.31
2	33	2.06
3	49	3.06
4	63	3.94
5	77	4.81
7	92	5.75
10	170	10.63
12	184	11.50
k [bar cm⁻¹ µl⁻¹ min]		0.963149

Anteil THF [% Porogen]	Steigung k [bar cm⁻¹ µl⁻¹ min]
0.0%	0.00871
3.3%	0.00830
6.7%	0.00611
10.0%	0.07460
13.3%	0.96315

Lichtmikroskop: 55 Skalenteile: 450 µm   1 Teil → 8 µm

M11_1 einzelne Poren ~3µ
sehr porös.

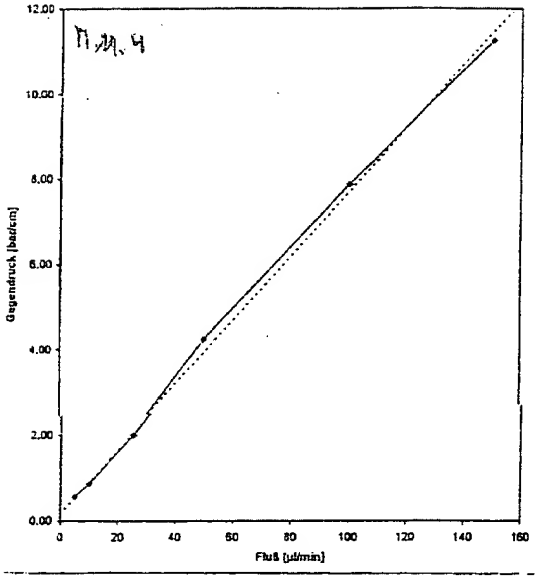
M11_2 große Poren, recht gleichmäßig

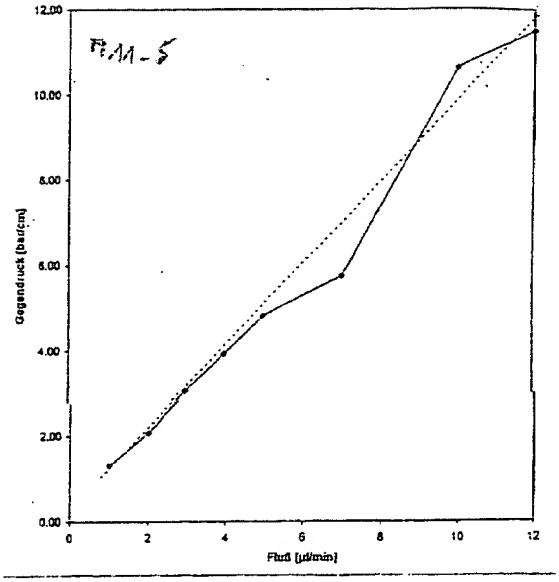
M11_3 große Poren, einzelne Klumpen (µm-Gruppen)

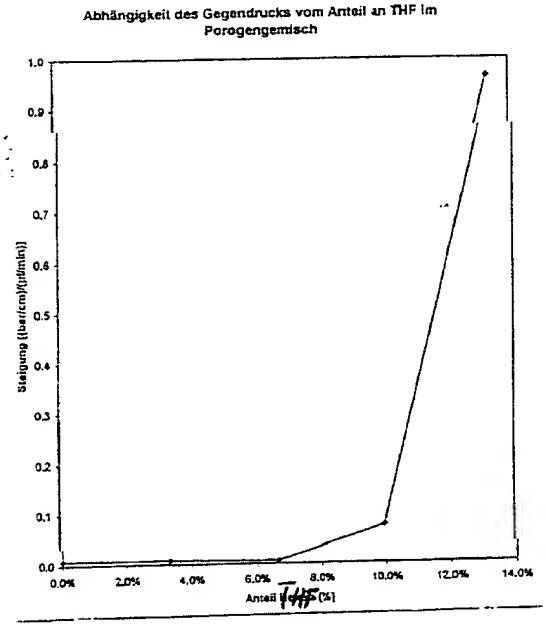
M11_4 sehr dicht, keine Risse, poren, die nicht gut zu sehen

M11_5 keine Clusterstruktur zu erkennen.

ol







Die nächsten Bilder zeigen M.M.4 und M.M.5 jeweils nochmals.

Für HPLC M.M.4 und M.M.5 testen.

25.08.98

JM 11.5 vom 6.8.98

kein Spülen 240bar / 5 µl/min

File. AP80875.SMP
GYNKOSOFT

SYKAM. 130 µl/min → Split → 4.6 µl/min
2 min 11sec / 10 µl
2 min 30sec / 4 µl/min

Equilibrium:
(A) H_2O, 0.1% TFA
(B) ACN, 0.1% TFA
50% A, 14.50 –

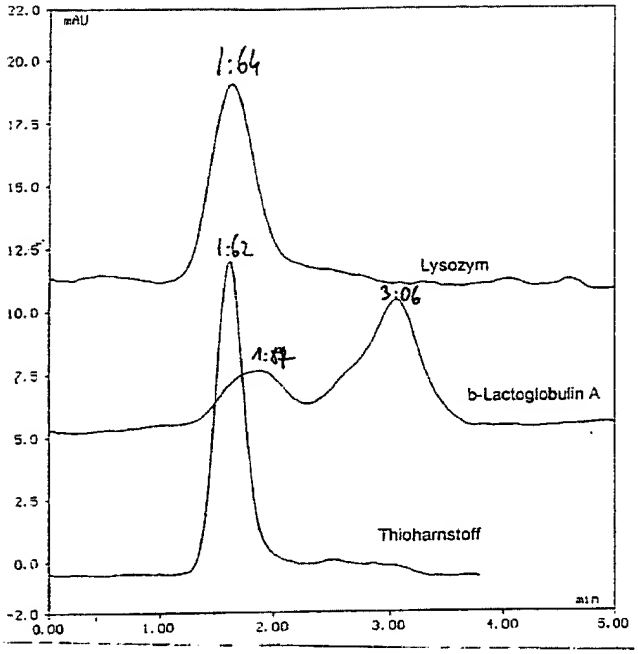
Edelstahl - T-Stück statt regulärem T-Stück
10 µl, 2 min 15sec $\frac{10}{2.25}$ 4.44 µl/min

Basislinie:
Thioharnstoff 0.05% in H_2O 50% ACN, 0.1% TFA
p ≙ 200 bar

100% H_2O, 0.1% TFA: Proteine zeigen keinen Peak → klebt an Kapillare?
Thioharnstoff nach ca. 1.50 min
50% ACN, 0.1% TFA: Proteine gleichzeitig mit Thioharnstoff: keine Retention
RBA

27.8.98
30% ACN, 0.1% TFA: Protein zurückgehalten, eluiert als Peak.
LAC A.
LYS keine Retention

Nun 40% ACN



50% ACN, 0.1% TFA
je 2mg/ml Protein
Retention von LACA bei 50% ACN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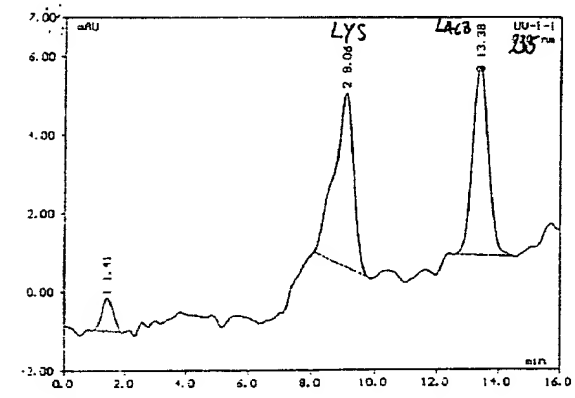
DP.Integration              SYS1 - C:\A980825.SMP                    Page   2
PSDVB 100x0.32mm, C12OH/THF-Porogen, Mil_5 98086                 1998-08-27/20:13

LysLacB 1mg/ml                        UV-1-1                      1998-08-27
Modified, H2O/30-60%ACN/0.1%TFA,4.5/130ul/min,25°C              GynkoSoft V5.50

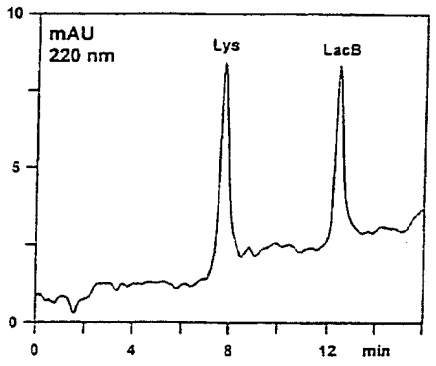
Smp. No/Pos: 35/1          Control:                  Standard:   --------
Sample Type: Integration   Signals: ANDI1.SIG        Inject:     20.0 ul
Acquisition: 1998-08-27/19:53 Report:                Dil. Fact.: 1.00000
Method:      DEFAULT.IPT   P-Table:                  Weight:     1.00000
```



No.	Ret.Time min	Type	Area mAU*min	Height mAU	Half Width min	Base Width min	Plates
1	1.418	MB	3.351e-1	0.84	0.415	0.720	44
2	9.060	BMB	3.170e+0	4.46	0.627	0.972	1156
3	13.379	BMB	2.320e+0	4.82	0.567	0.388	3080
---	-------	---	5.445e+0	10.12	--------	--------	--------

Endgradient Proteintrennung:
Lys, LacB je 1mg/ml, 20ml Inj.
30 - 60% ACN / 15min, 0.1% TFA
4.5 / 130µl/min
215 nm

A980825 -36



Separation of proteins in a monolithic capillary column

Column, PS-DVB (monolith, 100 x 0.32 mm); chromatographic conditions, mobile phase, (A) H_2O, 0.1% TFA, (B) ACN, 0.1% TFA, linear gradient, 30-60% B in 15 min; flow rate, 4.5 µl min^{-1}; temperature, 25 °C; detection, UV, 220 nm; sample, lysozyme, β-lactoglobuline B, 20 ng each.

08021999

Versuch einer Trennung von Oligonukleotiden im Monolithen M13_5

M13-5

l = 82 mm, id = 200 µm

Eluenten: A: 50 mM TEAA pH 6.8

B: 50 mM TEAA 20% ACN pH 6.8

Temperatur: 50°C

Splitkapillare TSP025375, 6 cm   Fluss 120/3.3 µl/min / 94 bar

Datei: A990209.SMT

Trennung von dT_8, dT_{16}

Testlauf mit Gradient 0–100% B/10 min. 0.11 min = 6.6 s

Trennung von dT_{12-18}

verschiedene Gradienten versucht.

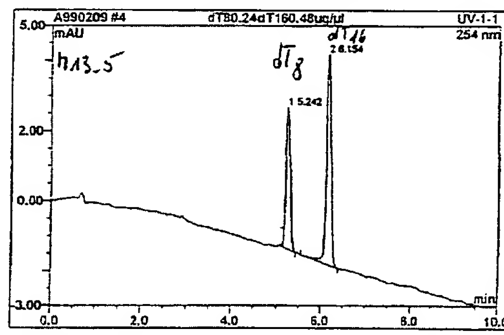
gute Trennung: 30–50% B/10 min

6 – 10% ACN/10 min

Operator:c72551 Timebase:A990209 Sequence:A990209 Page 4-1

10.2.1999 2:35 PM

4	dT80.24dT160.48µg/µl		
0-100%B/10min;A:50mMTEAApH6.8,B:50mMTEAA20%ACNpH6.8;120/3.3µl/min;0:2min;50°C			
Sample Name:	dT80.24dT160.48µg/µl	Injection Volume:	20.0 nl
Control Program:		Channel:	UV-1-1
Quantif. Method:	OLIGO1	Recording Time:	09.02.99 19:00

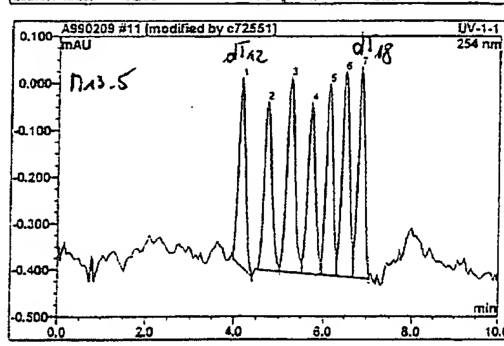


No.	Ret.Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	5.242	0.450	4.053	0.106	13593	1.303
2	6.154	0.744	6.008	0.111	16926	1.331
Total:		1.194	10.061			

Operator:c72551 Timebase:A990209 Sequence:A990209 Page 11-1

10.2.1999 2:34 PM

11	dT12-18 0.25µg/µl		
30-50%B/10min;A:50mMTEAApH6.8,B:50mMTEAA20%ACNpH6.8;120/3.3µl/min;0:2min;50°C			
Sample Name:	dT12-18 0.25µg/µl	Injection Volume:	20.0 nl
Control Program:		Channel:	UV-1-1
Quantif. Method:	OLIGO1	Recording Time:	09.02.99 21:29



No.	Ret.Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	4.158	0.075	0.405	0.175	3142	1.050
2	4.707	0.071	0.384	0.178	3889	1.551
3	5.224	0.088	0.420	0.192	4101	1.248
4	5.709	0.073	0.370	0.180	5552	1.084
5	6.122	0.082	0.412	0.189	5813	n.a.
6	6.483	0.086	0.441	0.182	7042	n.a.
7	6.835	0.082	0.454	0.171	6886	n.a.
Total:		0.556	2.888			